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Exploring microglial functions under physiological and pathological conditions by genome-wide expression profiling

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Chapter 7: Alpha-B-crystallin induces an immune-regulatory and antiviral microglial response in pre-active multiple sclerosis lesions

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Abstract

Microglial nodules are frequently observed in the normal appearing white matter of multiple sclerosis (MS) patients. Previously, we have shown that these clusters, which we call “pre-active MS lesions,” are closely associated with stressed oligodendrocytes and myelin sheaths that contain markedly elevated levels of the small stress protein alpha-B-crystallin (HspB5). Here, we show that microglia in these lesions express the recently identified receptors for HspB5, that is, CD14, Toll-like receptor family 1 and 2 (TLR1 and TLR2), and several molecular markers of the microglial response to HspB5. These markers were identified by genome-wide transcript profiling of 12 primary human microglial cultures at 2 time points after exposure to HspB5. These data indicate that HspB5 activates production by microglia of an array of chemokines, immunoregulatory mediators, and a striking number of antiviral genes that are generally inducible by type I interferons. Together, our data suggest that pre-active MS lesions are at least in part driven by HspB5 derived from stressed oligodendrocytes and may reflect a local attempt to restore tissue homeostasis.

Introduction

The pathologic hallmark of multiple sclerosis (MS) is the recurrent development of inflammatory demyelinating lesions in the CNS. In addition, normal-appearing white matter (NAWM) of MS patients shows evidence of both diffuse and focal abnormalities that are also likely relevant to the progression and clinical manifestations of MS (Banati *et al.* 2000; Miller, Thompson and Filippi 2003; Moll *et al.* 2011; Filippi *et al.* 2012). Among the abnormalities found in MS NAWM are clusters of activated microglia; these are present in the absence of overt demyelination, obvious leukocyte infiltration, or clear blood-brain barrier breakdown (van der Valk and Amor 2009). Their activated state is typically associated with elevated levels of expression of major histocompatibility complex class II molecules. These groups of activated microglia have previously been referred to as “earliest lesion” (Sanders, Conrad and Tourtellotte 1993), “type I lesion” (Gay *et al.* 1997), “preactive lesion” (De Groot *et al.* 2001), “newly forming lesion” (Barnett and Prineas 2004), early stages of a “pattern III lesion” (Marik *et al.* 2007), “microglial nodules” (Moll *et al.* 2011), and “prephagocytic lesion” (Haider *et al.* 2011). In the present study, we use the term “preactive lesion” to describe them.

When they are present close to actively demyelinating lesions, activated microglia seem to be associated with axonal pathology (Moll *et al.* 2011; Singh *et al.* 2013). Therefore, they may reflect tissue disturbances that directly result from the nearby inflammatory process. Conceivably, Wallerian degeneration of axons extending from actively demyelinating lesions and/or oligodendrocyte disturbances in the peri-plaque white matter could contribute to microglial activation in this context. At very similar frequencies, however, clusters of activated microglia are also found in NAWM distant from active lesions and well away from blood vessels. Such pre-active lesions appear to form in close association with oligodendroglial pathology, including apoptosis, or degeneration of oligodendrocyte processes (Barnett and Prineas 2004; Marik *et al.* 2007). In line with these latter findings, we previously documented the consistent presence in pre-active lesions of stressed oligodendrocytes that express high levels of the small heat shock protein alpha-B-crystallin (HspB5) (van Noort *et al.* 2010).

In the present study, we examined in more detail the possibility that oligodendroglial HspB5 contributes to microglial activation in pre-active MS lesions. Although the fact that HspB5 can activate cultured microglia already hinted to this possibility (Bsibsi *et al.* 2013), we now provide more evidence that this occurs in pre-active MS lesions. We show that the recently identified cellular receptors required for HspB5-mediated activation, that is, CD14 and toll-like receptors 1 and 2 (TLR1 and TLR2) (van Noort *et al.* 2013), are expressed by microglia in pre-active MS lesions. To characterize the microglial response to HspB5 that could be triggered through these receptors, we analyzed the response induced by

HspB5 in 12 primary human microglial cultures by genome-wide transcript profiling at 1 and 4 hours after activation. This comprehensive analysis of HspB5-induced transcript changes indicates that the HspB5-induced response in human microglia is dominated by immune-regulatory mediators and antiviral factors that are characteristic of a type I interferon (IFN)–induced cellular response. Having thus obtained not only a functional response profile, but molecular markers for the HspB5-induced microglial response as well, we subsequently confirmed the expression of several of these markers in pre-active MS lesions. Together, these findings strongly suggest that pre-active MS lesions reflect a beneficial immune-regulatory and antiviral response in microglia that is at least in part triggered by HspB5 from stressed oligodendrocytes.

Materials and methods

Brain Samples

Postmortem brain tissue samples were obtained from patients without neurologic disorders and patients with MS. The rapid autopsy regimen of the Netherlands Brain Bank in Amsterdam (coordinator Dr. I. Huitinga) was used to acquire the samples, with the approval of the medical ethical committee of the VU Medical Center. All patients and control donors had given informed consent for autopsy and the use of their brain tissue for research purposes. Tissue samples from subcortical white matter were obtained from control cases to isolate and culture microglia. For pathology studies, the tissues were either snap frozen in cooled isopentane and stored in liquid N₂ or fixed in 10% formalin and paraffin embedded. Classification of lesion stages was based on immunohistochemical detection of major histocompatibility complex class II/HLA-DR and the presence of myelin proteolipid protein to reveal areas of NAWM.

For all experiments, we used sterile clinical-grade recombinant human HspB5 (Delta Crystallon BV, Leiden, The Netherlands), containing 5 ng/mg (0.0005%) *Escherichia coli* protein, less than 0.7 EU/mg endotoxin, and less than 75 pg/mg bacterial DNA. The fraction of HspB5 that was contained in the appropriate multimeric (>400 kd) complexes was verified by size exclusion chromatography at more than 99.8%. The recombinant protein preparation did not activate HEK293 reporter cells expressing any of the individual human TLRs 1 to 10 (without CD14) at concentrations up to 200 µg/mL, confirming the absence of any confounding bacterial contaminants, as previously documented (13). Stimulation of cultured microglia was performed with 50 µg/ml recombinant HspB5 in all cases.

Immunohistochemistry

Paraffin sections were deparaffinized with xylene, rehydrated through graded alcohol, and washed in distilled water. Antigen retrieval was performed using citrate buffer and heating, and after washing, endogenous peroxidase activity was quenched by incubating the slides in 0.3% (wt/vol) H₂O₂ in methanol. Sections were blocked with PBS containing 1% (vol/vol) normal human serum and incubated overnight with the primary antibody diluted in PBS/serum. For immunofluorescence studies, snap-frozen sections (5 µm) were fixed with acetone. After rinsing in PBS, sections were incubated with a blocking solution (CleanVision IHC/ICC, Immunologic, Duiven, The Netherlands) supplemented with 10% (vol/vol) normal human serum (Dako, Glostrup, Denmark) for 2 hours. Subsequently, the

sections were washed in PBS and incubated overnight with primary antibodies at 4°C. After washing in PBS, sections were incubated with secondary antibodies goat anti-mouse IgG1 Alexa 594 and goat anti-rat IgG Alexa 488 (Invitrogen, Bleiswijk, The Netherlands) for 60 minutes at room temperature. Omission of primary antibodies served as negative controls. After washing in PBS, brain sections were incubated with 0.5% (wt/vol) Sudan black (Sigma, St. Louis, MO), and cell nuclei were visualized with 4',6-diamidino-2-phenylindole. Sections were viewed using confocal laser scanning microscopy (Leica TCS-SP confocal). Optical slices were collected in the z-direction. Images were collected using a Plan-Apochromate Leica and 63- or 100-fold objective. Image processing was performed using National Institutes of Health ImageJ software.

Isolation and Culturing of Human Microglia

Human adult microglia were isolated and cultured from fresh postmortem brain samples, as previously described (Bsibsi *et al.* 2013). Briefly, tissue samples were dissected from subcortical white matter, and visible blood vessels were removed. After a 20-minute digestion in 0.25 % (wt/vol) trypsin (Sigma), the cell suspension was gently triturated and washed with DMEM/HAM-F10 medium containing 10% (vol/vol) fetal calf serum and antibiotic supplements. After passage through a 100-µm filter, myelin was removed by Percoll gradient centrifugation. Erythrocytes were lysed by 15-minute incubation on ice with 155 mmol/L NH₄Cl, 1 mmol/L KHCO₃, and 0.2% (wt/vol) bovine serum albumin. Recombinant human granulocyte-macrophage colony-stimulating factor (PeproTech Inc., Rocky Hill, NJ) was added to microglial cultures every 3 days at a final concentration of 20 µg/mL to promote proliferation and survival. The purity of the cultured microglia was verified by immunostaining for CD68 before cell stimulation and RNA isolation. Generally, microglia cultures were found to be more than 99% pure.

Microarray Transcript Profiling

Total macrophage RNA was isolated in 0.75 mL of TRIzol and extracted according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). RNA integrity and concentrations were measured on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a Nanodrop spectrophotometer ND-1000 (Fisher Scientific, Waltham, MA). All samples were of high quality with an RNA Integrity Number (RIN value) between 7.6 and 8.7 and 260/280 ratios greater than 2.00. Samples of 500 ng total RNA were used as input for amplification and labeling with the Quick Amp Labeling kit (Agilent Technologies, Palo Alto, CA), according to the manufacturer's guidelines, including control spikes. Labeled

RNA was purified using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) yielding 7.5 µg or more of labeled cRNA and specific activities greater than 15.3 pg Cy3 dye per microgram cRNA and 18.9 pg Cy5 dye per microgram cRNA. Labeled samples were hybridized onto whole human genome GE 4 × 44K GF4112F microarrays according to the manufacturer's protocol (Agilent Technologies). Scanning was performed using a microarray scanner G2505C (Agilent Technologies) and Feature Extraction v10.7 using the manufacturer's protocols. The microarray data have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus database (database number, GSE30658) (<http://www.ncbi.nlm.nih.gov/geo>).

Real-time Polymerase Chain Reaction

Total cellular RNA was isolated using TRIzol as previously described. RNA was reverse transcribed into cDNA, and levels of different genes, including β-actin as a reference, were determined by real-time polymerase chain reaction (RT-PCR) using SYBR green.

Statistical Analyses

Preprocessing and analysis of microarray data ($n = 12$) were performed with R (16) and BioConductor package Limma (Smyth 2005). Background correction was performed with the normexp function with an offset value of 50. Global Loess within array normalization and quantile between array normalization were performed (Ritchie *et al.* 2006). Array quality weight was used to fit a heteroscedastic model to the expression values for each gene and was incorporated into the linear model approach to find differentially expressed genes (19). A Benjamini-Hochberg corrected value of $p < 0.05$ and a fold change greater than 2.0 were used as cutoff to determine significance.

Cytokine and Chemokine Quantitation

Quantitation of cytokines and chemokines in microglial culture supernatants was performed using commercially available ELISA kits according to the manufacturer's instructions (eBioscience, San Diego, CA; Sanquin, Amsterdam, The Netherlands).

Results

Transient Colocalization of HspB5 and Microglial Receptors in Preactive MS Lesions

Recently, we identified CD14, TLR1, and TLR2 as the 3 receptors required for a cellular response to extracellular HspB5 (Bsibsi *et al.* 2013). With the TLR1/2 complex as the signaling element and CD14 acting as an essential co-receptor for HspB5, cellular responsiveness to HspB5 is thus limited to macrophage-like cells that express all 3 receptors. To examine whether microglia in pre-active MS lesions could potentially be HspB5-responsive cells, we examined the expression of CD14, TLR1, and TLR2 in pre-active MS lesions. As illustrated in Figure 7.1, clusters of activated microglia, typified by elevated expression of HLA-DR, consistently expressed high levels of immunoreactivity for all 3 pattern-recognition receptors. Other glial cells or microglia outside pre-active MS lesions did not or did at much lower frequency. In healthy white matter from control subjects, expression of CD14, TLR1, or TLR2 was not detectable, consistent with previous data (Bsibsi *et al.* 2002).

In a previous study, we documented selective expression of HspB5 by stressed olig-2–positive oligodendrocytes in pre-active MS lesions (Figure 7.2A) and co-localization of such oligodendroglial HspB5 with HLA-DR on neighboring microglia (van Noort *et al.* 2013). Apart from co-localization between HspB5 and HLA-DR at contact points between the oligodendrocyte and microglial cell surface, such co-localization was also found in intracellular microglial vesicles, suggestive of low-level ingestion of HspB5-containing myelin or oligodendrocyte-derived exosomes. Given such signs of intimate contact between oligodendroglial HspB5 and microglial HLA-DR, we examined pre-active lesions for signs of additional colocalization between HspB5 and microglial TLR2. Whereas this was indeed occasionally found, co-localization was not widespread (Figure 7.2B, C). This relative paucity of co-localized HspB5 and TLR2 in pre-active lesions may be explained by the only transient nature of their association, as revealed by in vitro modeling of the interaction. After addition of HspB5 to cultured human microglia, co-localization of HspB5 with TLR2 in endosomal vesicles became visible within 10 minutes but rapidly vanished again within 1 hour (Figure 7.2D). Apparently, processing of HspB5 on internalization into HLA-DR–positive vesicles leads to a rapid loss of its detectability by immunostaining.

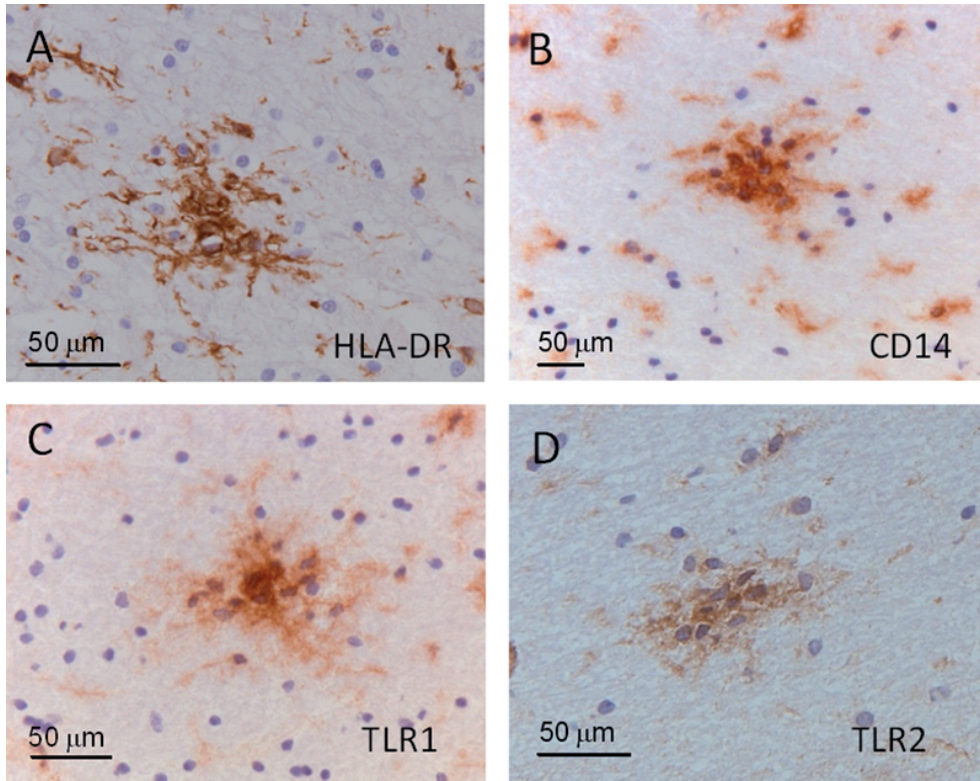


Figure 7.1: Microglia in pre-active MS lesions. (AYD) Expression of HLA-DR (A) and the HspB5 receptors CD14 (B), Toll-like receptor 1 and 2 (TLR1 [C] and TLR2 [D]) in pre-active MS lesions. This immunoreactivity was not found elsewhere in MS normal-appearing white matter or in white matter from non - MS control samples.

Transcript Profiling of HspB5-Activated Microglia

The above data clarify that, in pre-active MS lesions, HspB5 derived from stressed oligodendrocytes and myelin would be available for direct interaction with CD14 and TLR1/2 on surrounding microglia. To explore the nature of the cellular response that could thus be triggered, we performed genome-wide microarray-based transcript profiling of 12 different primary human microglial isolates after stimulation with HspB5. RNA was collected at 1 and 4 hours after activation and processed for genome-wide microarray transcript profiling. In this way, a robust and comprehensive data set was obtained to document the changes induced in microglia by HspB5. The most prominent changes in levels of transcripts seen in each of the 12 microglia cultures are reflected by heatmaps shown in Figure 7.3. They confirm a high level of consistency in the data sets obtained for the 12 cultures.

After 4 hours of activation, significantly altered levels of transcripts (i.e. with a more than 2-fold change in expression levels) were recorded for 537 genes ($p < 1E-3$). Of these, 488 genes were induced whereas 49 were down-regulated. Data for the 50 transcripts that were induced the strongest at 4 hours are summarized in Figure 7.4. In the group of microglial genes, the expression of which was induced the strongest by HspB5 after 4 hours, several functional categories can be discerned. The dominant categories, in terms of the number of top 50 genes representing each category, are 1) genes that control cellular movement, 2) genes that control inflammation, and 3) type I IFN-inducible antiviral genes.

Many of the dominantly activated genes are chemokines, including *CCL4*, *CCL5*, *CCL19*, *CCL20*, *CXCL1*, *CXCL2*, *CXCL3*, *CXCL8*, and *CXCL10* (also known as interferon- γ -induced protein 10 [IP-10]). Some of these may promote self-recruitment of microglia and thus may well contribute to the clustering of microglia seen in pre-active MS lesions. For example, *CCL19* was induced 6-fold, whereas its receptor *CCR7* (CD197) was induced 15-fold; however, other chemokines seem to be aimed at different cell types.

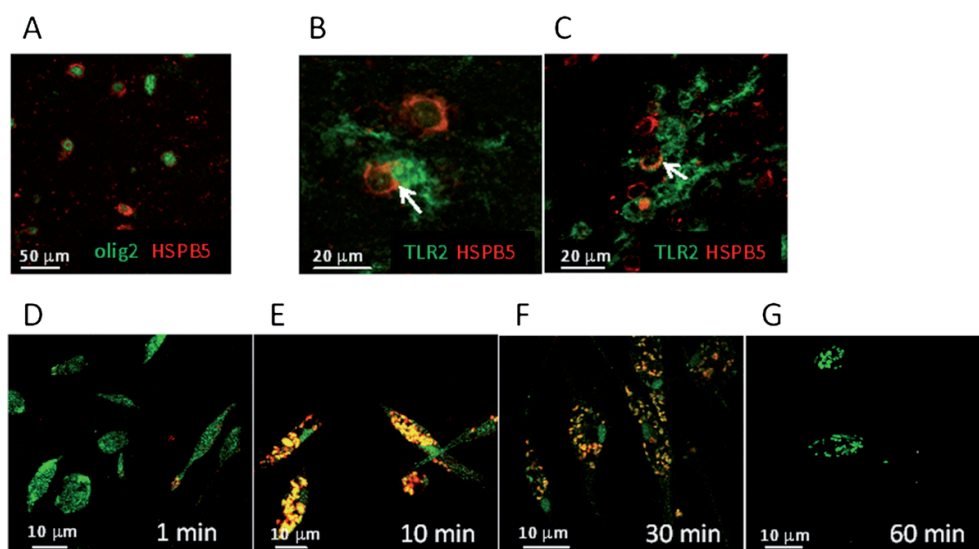


Figure 7.2: Co-localization of HspB5 and its signaling receptor toll-like receptor 2 (TLR2) in microglia. HspB5 in pre-active MS lesions, exclusively expressed by olig-2Y positive oligodendrocytes (A), can be found co-localized with microglial TLR2 but only occasionally (B, C). (D–G) The relative paucity of this co-localization in vivo likely results from the transient nature of the HspB5-TLR complex, as demonstrated by examining the fate of recombinant HspB5 added to cultured human microglia. After uptake, co-localization of HspB5 with microglial TLR2 peaks at 10 minutes to rapidly fade again.

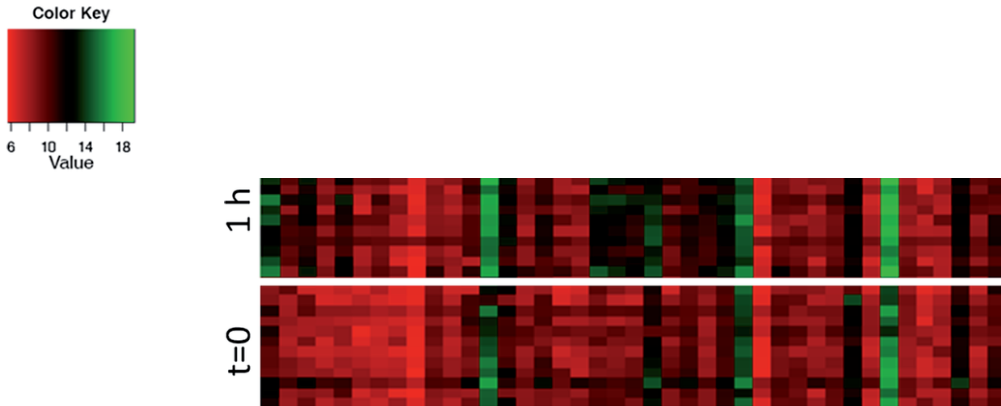


Figure 7.3: Heatmap representation of the most prominent transcript changes in human microglia 1 or 4 hours after activation with HspB5. The heatmaps provide an illustration of the early signal changes for the 50 most prominently induced transcripts in 12 different microglial cultures, as examined by genome-wide microarray profiling.

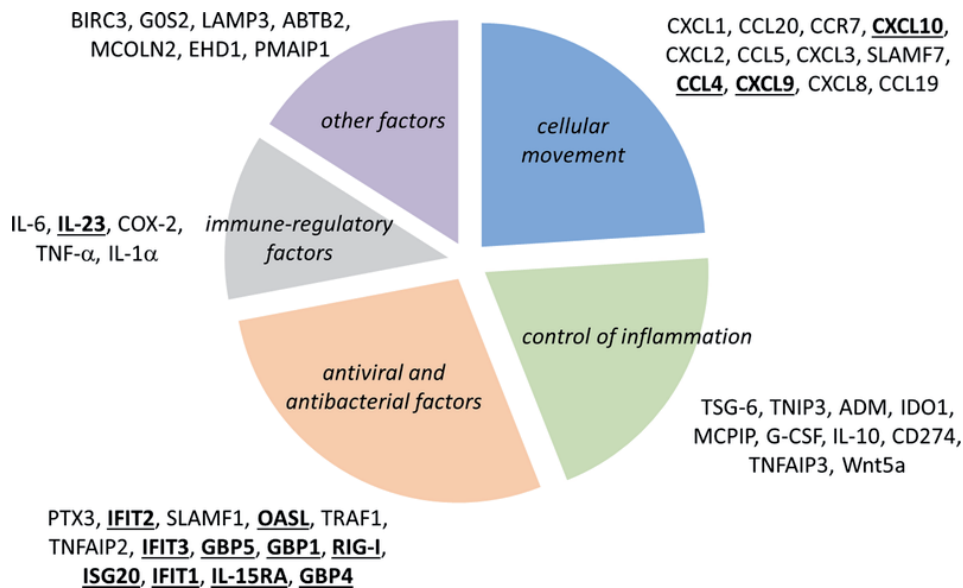


Figure 7.4: Summary and functional annotations of the 50 most prominently induced transcripts in cultured human microglia 4 hours after activation by HspB5. Microglial

transcripts that are bold and underlined have previously been documented as type I interferon inducible gene products.

For example, there was strong induction of *CXCL1*, *CXCL2*, *CXCL3*, and *CXCL8*, all of which target CXCR2. This chemokine receptor fulfills several different functions within the CNS, including recruitment of oligodendrocyte progenitor cells (Veenstra and Ransohoff 2012). Although CXCL10 may be important for leukocyte recruitment, it also plays complex roles in a CXCL10/CXCR3-controlled network of glia-glia and glia-neuron interactions within the CNS, possibly contributing to maintenance of homeostasis (van Weering *et al.* 2011; Olah *et al.* 2012).

This duality in possible functional impact also applies to immune-regulatory factors that were induced, including interleukin 6 (*IL-6*), cyclooxygenase 2 (*COX-2*), and tumor necrosis factor (*TNF*). These mediators are often labeled as “proinflammatory” factors but are, in fact, well known to support protective functions as well. Another abundantly induced transcript was that encoding IL-1 β ; however, no IL-1 β protein was detectable in the supernatants of microglia cultures (Figure 7.5). The ability of our microglial cultures to produce and secrete IL-1 β was confirmed by activating the cells with lipopolysaccharides in place of HspB5 (data not shown). Clearly, HspB5 strongly induces IL-1 β transcripts but, unlike stimulation with lipopolysaccharides, it fails to activate caspase-mediated maturation required for secretion of active IL-1 β , similar to what we previously observed for human macrophages (van Noort *et al.* 2013).

Several genes with well-documented functions in controlling inflammation form a second dominant group of HspB5-activated microglia genes. These include *IL-10* (Sabat *et al.* 2010), *CD274* (also known as B7-H1 or PD-L1) (Fife and Pauken 2011), *TNFAIP6* (also known as TSG-6) (Milner, Higman and Day 2006), *TNFAIP3* (also known as A20) and its binding partner *TNIP3* (Verstrepen *et al.* 2009; Ma and Malynn 2012), indoleamine 2,3-dioxygenase (IDO1) (Cesario, Rocca and Rutella 2011), adrenomedullin (Cheyuo, Yang and Wang 2012), *WNT5A* (Varela-Nallar *et al.* 2012), granulocyte-colony stimulating factor (Maurer, Schäbitz and Schneider 2008), and zinc finger CCCH-type containing 12A (*ZC3H12A*, also known as MCPIP) (Niu *et al.* 2011). Particularly, detailed documentation exists on the ability of IL-10, CD274, TSG-6, TNFAIP3, and IDO1 to suppress destructive inflammatory processes, promote the recruitment and activation of regulatory elements in the T-cell repertoire, and stimulate the restoration of self-tolerance in the immune system. That these factors are induced in concert strengthens the notion that an important component of the HspB5-induced microglial response is indeed immune suppressive. Additional factors that are induced by HspB5 in microglia can be seen in this

context as well; for example, the long pentraxin 3 (induced more than 30-fold), which prevents neutrophil infiltration (34).

A final major and unexpected group of HspB5-induced factors include type I IFN-inducible genes that have marked antiviral activities. They include 3 IFN-induced proteins with tetratricopeptide repeats (IFITs), *IFIT1*, *IFIT2*, and *IFIT3*, and DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (*DDX58*), more commonly known as the intracellular antiviral pattern-recognition receptor RIG-I (retinoic acid-inducible gene 1). Additional members of this group are 2'-5'-oligoadenylate synthetase-like (OASL), the IFN-inducible guanylate binding protein (GBP) 1, 4, and 5, and IFN-inducible 3'-5' exonuclease (*ISG20*). Together, these type I IFN-inducible genes represent an impressive arsenal of antiviral factors that can initiate a cellular response to viral RNA (Stark; Waddell *et al.* 2010; Schoggins *et al.* 2011).

The prominent induction of both subunits of IL-23, viz. *IL23A* and *IL12B*, also falls into the category of type I IFN-inducible genes. Interleukin 23 is an important regulatory cytokine that is well known to promote Th17 responses. However, because pre-active MS lesions are devoid of infiltrated lymphocytes, HspB5-induced production of IL-23 likely targets microglia. As an autocrine stimulatory factor, IL-23 can induce production of microglial IL-17 which, in turn, promotes activation of astrocytes and blood-brain barrier breakdown (Lv *et al.* 2011). In this way, IL-23 can indirectly promote leukocyte recruitment into the CNS. An autocrine function of IL-23 is supported by the lack of any significant induction of IL-17 transcripts at 1 or 4 hours after HspB5-mediated activation, whereas IL-17 protein markedly accumulates in the culture media of microglia after 24 hours (van Noort *et al.* 2013). Therefore, IL-17 production is not likely the direct result of HspB5-mediated activation but the indirect result of IL-23 signaling, which is, indeed, rapidly secreted by HspB5-activated microglia (Figure 7.6).

Although HspB5 also leads to the suppression of several genes, their numbers are limited and the changes in their expression are much less prominent than those of activated genes. Unfortunately, insufficient functional data have so far been reported on these factors to allow for a meaningful interpretation of their possible biologic relevance in the current context.

Validation of Microarray Data

The microarray-based data for several genes of interest were validated by RT-PCR analyses of 8 samples from the collection of microglial RNA samples used for microarray profiling (Figure 7.5). In all cases, RT-PCR analysis of samples collected at 4 hours confirmed the marked increase changes in transcript levels of selected migratory, immune-regulatory,

and antiviral factors. Because of the higher sensitivity and lesser robustness of RT-PCR assays, as compared with microarray profiling, however, the extent of induction as well as the inter-individual variation between samples was generally higher as compared with the microarray data.

Additional confirmation of the validity of the microarray data was obtained by analyzing protein levels in the culture supernatants collected after HspB5-mediated activation of microglia (Figure 7.6). This analysis confirmed marked accumulation of IL-6, IL-10, IL-23, PTX3, and TNF during the first 4 hours, whereas IL-1 β was absent from all culture supernatants. As previously explained, whereas IL-1 β –encoding transcripts are strongly induced by HspB5, mature IL-1 β protein is not secreted.

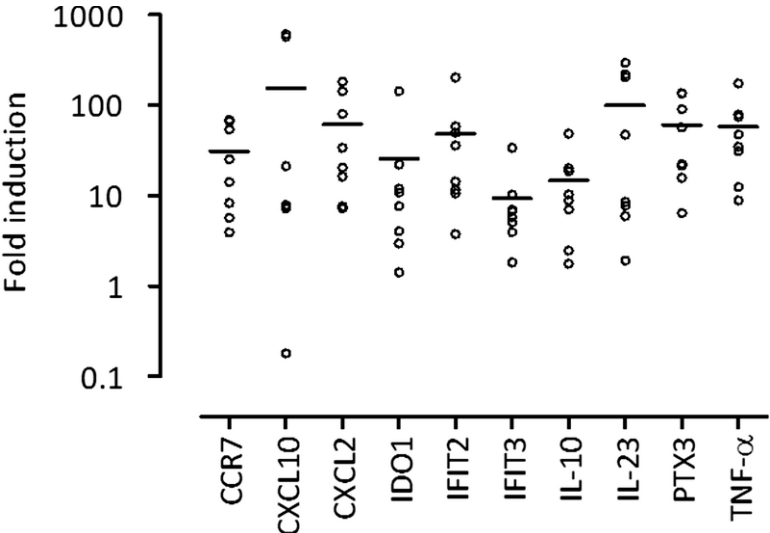


Figure 7.5: Real-time polymerase chain reaction analysis of transcripts encoding a selection of mediators induced in human microglia by HspB5. To validate the microarray data, 8 RNA samples from those collected after 4 hours and used for microarray profiling were additionally analyzed by RT-PCR. Levels of the various transcripts, normalized for A-actin, are expressed relative to those found in untreated microglial cultures of samples from the same donors.

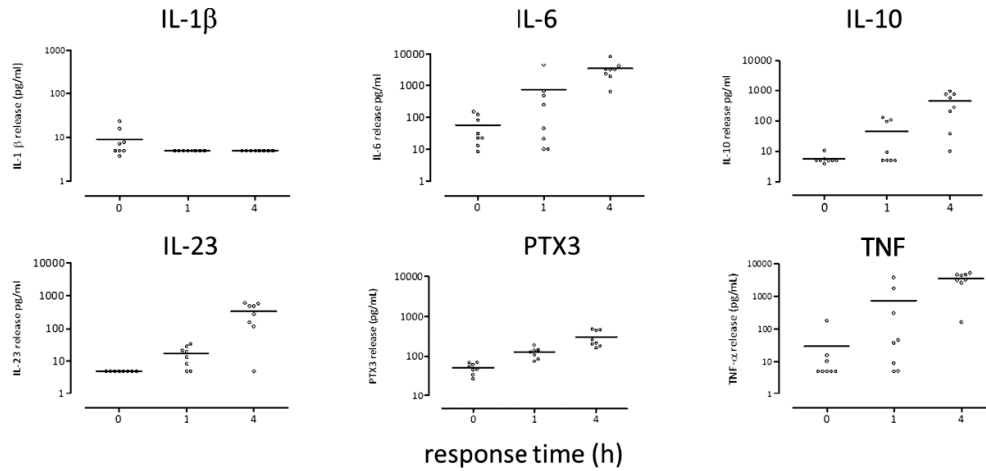


Figure 7.6. *HspB5*-induced secretion of various mediators into the microglial culture medium. To validate the microarray data, protein levels of various mediators were determined by ELISA in the microglial culture medium obtained after 4 hours of stimulation with *HspB5*.

Expression of Type I IFN-Inducible Microglial Genes in Preactive MS Lesions

The above data not only reveal the functional phenotype of *HspB5*-activated microglia but also provide several markers to examine the microglial phenotype in pre-active MS lesions. Increased expression of the *HspB5* response markers TNF and IL-10 in pre-active MS lesions has been documented (van Horssen *et al.* 2012). To extend these findings and to maximize the discriminatory quality of these markers, we selected proteins that are more strongly induced by *HspB5* in human microglia than in human macrophages of which we recently profiled the response to *HspB5* (Bsibsi *et al.* 2014). Especially IL-23 and the type I IFN-inducible genes IFIT1, IFIT2, and IFIT3 and RIG-I qualify as such. After 4 hours of activation, IL-23A (the p19 subunit) is induced 47-fold in microglia and only 4-fold in macrophages. Interleukin 12B, the p40 subunit of IL-23, is induced 22-fold in microglia and not at all in macrophages. Similar differences apply to the 3 IFITs and RIG-I. None of these factors is induced more than 3-fold in macrophages, with IFIT1 being suppressed, whereas they are strongly induced in microglia. Therefore, IL-23, RIG-I, and the 3 IFITs were selected for screening of pre-active MS lesions. We found consistent and clearly detectable expression of these response markers in microglia within all pre-active MS lesions examined (Figure 7.7).

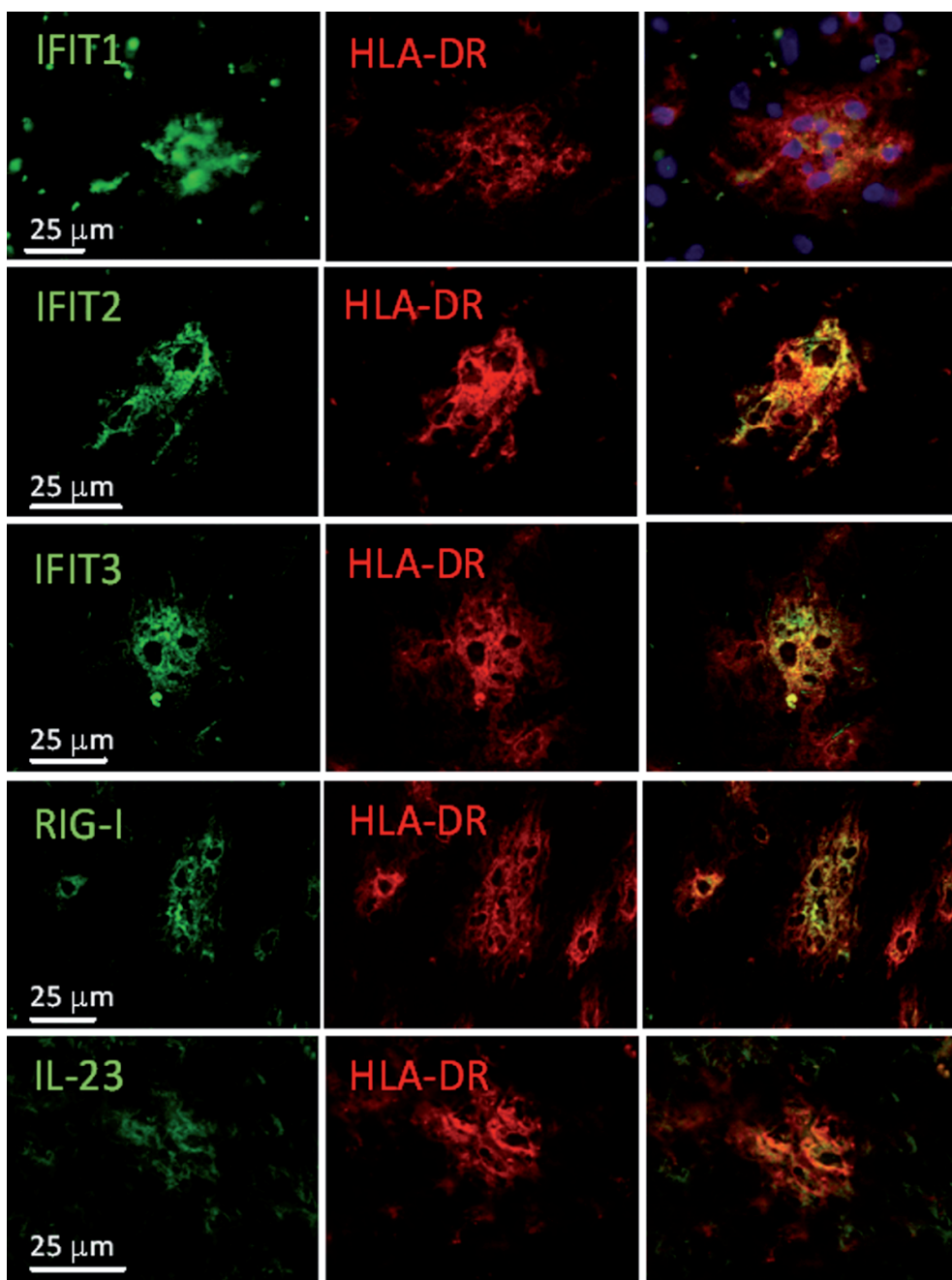


Figure 7.7: Expression of HspB5-induced microglial response markers in preactive MS lesions. By confocal laser scanning microscopy, expression was examined of various HspB5 response markers in HLA-DR⁺ positive microglial clusters in pre-active MS lesions.

Discussion

Although many studies have focused on the inflammatory demyelinating lesions that are the pathologic hallmark of MS, the additional disturbances that are present in NAWM of MS patients are much less well understood (1). A frequently documented phenomenon in NAWM is the occurrence of small clusters of activated microglia, expressing increased levels of HLA-DR molecules, benzodiazepine-binding sites, immune-regulatory cytokines such as TNF and IL-10, and free radical-generating enzymes (Bsibsi *et al.* 2013). Our present data strongly suggest that the appearance of these clusters of activated microglia, which we term 'pre-active MS lesions', involves at least in part a direct microglial response to HspB5 from stressed oligodendrocytes. They also indicate that the HspB5-induced state of microglial activation is typified by production of immune-regulatory and antiviral mediators and, therefore, likely reflects a local attempt to restore tissue homeostasis.

That the microglial response to HspB5 in pre-active MS lesions is a restorative response is supported by transcript profiling of cultured human microglia after activation by HspB5. Functional annotation of individual genes is not without complications, given the often dualistic or even multifunctional roles of many proteins. Yet, the likelihood that their biologic role is correctly interpreted considerably increases when many different mediators with similar functional annotations are all induced at the same time. In the case of HspB5-mediated activation of microglia, we found strong up-regulation of factors that are well known to suppress inflammation and to promote immune tolerance and tissue repair. These included *IL-10*, *CD274* (B7-H1 or PD-L1), *IDO1*, *TSG-6*, adrenomedullin, *MCPIP*, *WNT5A*, and *G-CSF* (Milner, Higman and Day 2006; Maurer, Schäbitz and Schneider 2008; Verstrepen *et al.* 2009; Cesario, Rocca and Rutella 2011; Fife and Pauken 2011; Niu *et al.* 2011; Cheyuo, Yang and Wang 2012; Ma and Malynn 2012; Varela-Nallar *et al.* 2012). Several other, similarly immune-suppressive and restorative, factors were induced at somewhat lower levels. In addition, HspB5 induced a striking number of factors that are generally inducible by type I IFNs. These included *OASL*, *IFIT1*, *IFIT2*, *IFIT3*, *RIG-I*, *GBP1*, *GBP4*, *GBP5*, and *ISG20*, all of which have well-documented antiviral functions (Waddell *et al.* 2010; Lv *et al.* 2011; Schoggins and Rice 2011). Alpha-B-crystallin additionally induced up-regulation of *IRF1* (4.22×), *IRF7* (2.53×), and *IRF8* (2.05×). This further supports the notion that a type I IFN-like antiviral response is also an important feature of the HspB5-triggered microglial response. It is tempting to relate this apparently type I IFN-like response induced by HspB5 to the therapeutic effects of IFN- β in MS patients as well.

Apart from these anti-inflammatory and antiviral mediators that are prominently expressed 4 hours after exposure to HspB5 in vitro, typical products of a TLR-activated NF- κ B/MAPK-driven response were also found, particularly at early time points. They

included *IL-6*, *TNF*, *COX-2*, and *CXCL8*, as well as negative feedback factors for NF- κ B/MAPK-mediated signaling, including, for example, *TNFAIP3*, *TNIP3*, and *IL-10*. The appearance of factors that are traditionally seen as proinflammatory, including *IL-6*, *TNF*, *COX-2*, and *IL-23* may seem to contradict the idea that the HspB5-induced microglial response in pre-active MS lesions is beneficial; however, it is important to point out that the functional roles of the above factors are complex. Under certain conditions, they do not promote inflammatory damage, but they also contribute to the control of inflammation and restoration of homeostasis. Interleukin 6 expression is associated with oligodendrocyte preservation during MS (Schönrock, Gawlowski and Brück 2000), TNF-mediated signaling contributes to ameliorating experimental autoimmune encephalomyelitis and MS (Gregory *et al.* 2012), and *COX-2* catalyzes the production of lipid resolvins and contributes to immune-suppressive IDO1 functions (Cesario, Rocca and Rutella 2011; Mancini and Di Battista 2011). The role of *IL-23* in the CNS is also still incompletely understood (van Weering *et al.* 2011). Contrary to expectations, for example, anti-*IL-23* antibodies do not suppress disease activity in MS patients (Segal *et al.* 2008; Vollmer *et al.* 2011). Clearly, labeling these mediators as “pro-inflammatory” is an oversimplification of their complex biologic functions.

The notion that the HspB5-induced state of microglial activation helps to suppress inflammation and promotes repair is additionally supported by several reports documenting a beneficial anti-inflammatory effect of HspB5 during neuroinflammation in vivo, including experimental autoimmune encephalomyelitis, stroke, and spinal cord injury (Masilamoni *et al.* 2006; Ousman *et al.* 2007; Arac *et al.* 2011; Klopstein *et al.* 2012). Fully in line with the above microarray data, therefore, microglial activation by HspB5 clearly leads to suppression of neuroinflammation in vivo. More recently, these findings have been extended by data demonstrating the anti-inflammatory effects of HspB5 also in other experimental models of inflammation (Pangratz-Fuehrer *et al.* 2011; Velotta *et al.* 2011; Park *et al.* 2013) in which HspB5 mediates its effects by activating immune-regulatory macrophages rather than microglia (van Noort *et al.* 2013).

In summary, activated microglia in NAWM of MS patients express both the receptors for HspB5 and the molecular signature of activation by HspB5. They are consistently accompanied by, and make direct contact with, stressed oligodendrocytes and myelin sheaths that contain high levels of HspB5. Together, our findings suggest that pre-active lesions reflect local efforts to resolve whatever injurious factor is responsible for the frequent induction of oligodendrocyte stress in NAWM during MS.

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